Lifelong imbalanced LA/ALA intake impairs emotional and cognitive behavior via changes in brain endocannabinoid system

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Abstract **Imbalanced dietary n-3 and n-6 PUFA content has been associated with a number of neurological conditions. Endocannabinoids are n-6 PUFA derivatives, whose brain concentrations are sensitive to modifications of fatty acid composition of the diet and play a central role in the regulation of mood and cognition. As such, the endocannabinoid system appears to be an ideal candidate for mediating the effects of dietary fatty acids on mood and cognition. Lifelong administration of isocaloric α-linolenic acid (ALA)-deficient and -enriched diets induced short-term memory deficits, whereas only dietary ALA enrichment altered emotional reactivity in adult male rats compared with animals fed a standard diet that was balanced in ALA/linoleic acid (LA) ratio. In the prefrontal cortex, both diets reduced 2-AG levels and increased MAG lipase expression, whereas only the enriched diet reduced AEA levels, simultaneously increasing FAAH expression. In the hippocampus, an ALA-enriched diet decreased AEA content and NAPE-PLD expression, and reduced 2-AG content while increasing MAG lipase expression. These findings highlight the importance of a diet balanced in fatty acid content for normal brain functions and to support a link between dietary ALA, the brain endocannabinoid system, and behavior, which indicates that dietary ALA intake is a sufficient condition for altering the endocannabinoid system in brain regions modulating mood and cognition.**—Zamberletti, E., F. Piscitelli, V. De Castro, E. Murru, M. Gabaglio, P. Colucci, C. Fanali, P. Prini, T. Bisogno, M. Maccarrone, P. Campolongo, S. Banni, T. Rubino, and D. Parolaro. **Lifelong imbalanced LA/ALA intake impairs emotional and cognitive behavior via changes in brain endocannabinoid system.** ***J. Lipid Res.*** **2017.** 58: **301–316.**

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Among nutrients that can play a role in improving human health, two distinct classes of essential fatty acids, n-6 and n-3, have received wide attention during the last decades. The n-6 and n-3 fatty acids are fundamental nutrients that must be ingested as foods or dietary supplements.

Linoleic acid (LA) and α-linolenic acid (ALA) are the parent fatty acids of the n-6 and n-3 groups, respectively. These 18-carbon fatty acids are required for the synthesis of longer PUFAs, such as arachidonic acid (AA), EPA, and DHA. Conversion of n-6 and n-3 fatty acids to PUFAs occurs as a result of enzymatic desaturation and elongation steps. In addition, because the enzymes that are involved in these mechanisms have the same functions in the two fatty acid groups, the n-6 and n-3 fatty acids compete for them for their biosynthesis; in experimental animals, it has been shown that formation of n-3 long-chain PUFAs (LCPUFAs) is dependent on LA and total dietary PUFA concentration. However, the conversion rate of ALA to EPA, and particularly to DHA, is very low in humans (1). Thus, while both n-6 and n-3 fatty acids are essential for health, the balance of the two is critical, as well as the dietary intake of n-3 LCPUFAs, EPA, and DHA (2) (**Scheme 1**).

Over the last decades, the ratio of n-6 to n-3 fatty acid intake in the overall population, including children and adolescents, has dramatically shifted in the Western diet. In the eating habits of Western industrial countries, food

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA-EA, docosahexaenoyl ethanolamide; Ef, exploring the familiar object; En, exploring the new object; EPA-EA, eicosapentaenoyl ethanolamide; FAME, fatty acid methyl ester; FID, flame ionization detector; FST, forced swim test; LA, linoleic acid; LCPUFA, long-chain PUFA; NOR, novel object recognition; PFC, prefrontal cortex; PND, postnatal day; PPI, prepulse inhibition.

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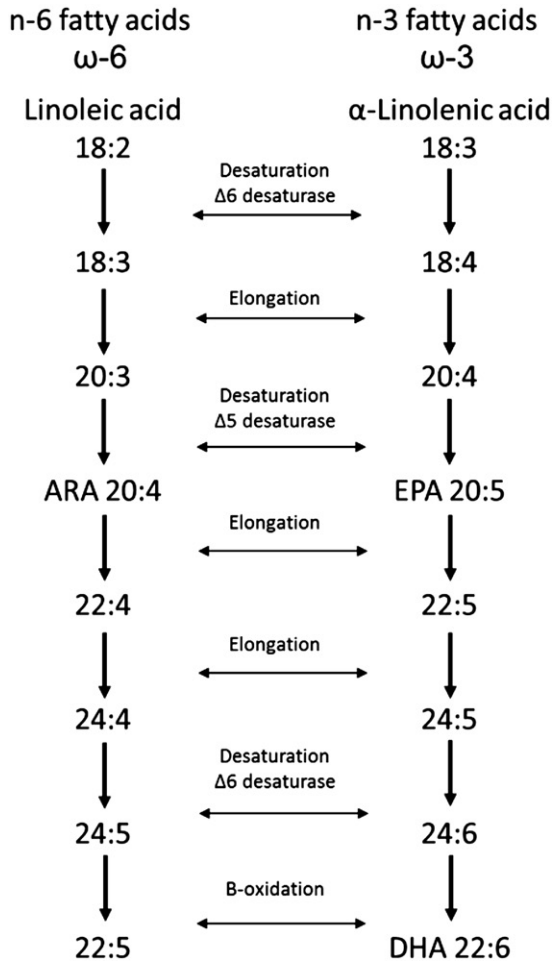
tends to be rich in n-6 fatty acids and low in long-chain n-3 fatty acids (3, 4). This dysregulation can affect the equilibrium of several organs and particularly of the brain that is highly enriched in LCPUFAs. Brain's LCPUFAs are involved in the maturation of neuronal structures and are essential throughout the entire lifespan for maintaining normal brain activities (5, 6). Consequently, it is unsurprising that a lack of n-3 LCPUFAs or an imbalance in the n-3 and n-6 ratio has been associated with a number of neurological and psychiatric disorders, including depression, anxiety, schizophrenia, and attention deficit hyperactivity disorder in both children and adults (7–12).

Despite the growing body of evidence suggesting a link between n-3 LCPUFA dysregulation and neuropsychiatric diseases, how dietary fatty acids can actually affect the brain and behavior is still poorly understood.

The endocannabinoid system appears to be an ideal candidate for mediating the effects of dietary fatty acids on mood and cognition. Indeed, endocannabinoids are local neuromodulators that are metabolic derivatives of the n-6 LCPUFA, AA, and their concentrations in the brain are sensitive to modifications of the fatty acid composition of the diet (13, 14). Furthermore, the endocannabinoid system plays a role in the regulation of mood and cognition under physiological conditions, and its dysregulation is believed to contribute to the development of several neuropsychiatric pathologies (15).



Scheme 1. Schematic representation of n-6 and n-3 metabolism.

In this context, endocannabinoids shape neuronal architecture (16), and recent preclinical studies comparing the effects of lifelong n-3-deficient to regular diets have pointed out that long-term ALA dietary insufficiency impairs endocannabinoid-mediated long-term synaptic depression in brain areas associated with the development of anxiety- and depressive-like behaviors (17, 18), suggesting that lifelong deficiency in n-3 PUFAs may influence cerebral areas controlling mood through alteration of the endocannabinoid system functionality. However, the above-cited studies investigated the consequences of diets deficient in ALA and without comparing the effects of such dietary imbalances to those present after administration of a healthy diet balanced in n-3 and n-6 fatty acid content.

The present study aimed at comparing the effects of lifelong administration of isocaloric diets enriched or deficient in ALA, but containing similar amounts of LA, to those of a standard diet, with a balanced and recommended n-3/n-6 ratio. On this basis, we investigated whether: *1*) imbalanced amounts of ALA in the diet could be a sufficient condition for the development of altered emotional and cognitive behaviors at adulthood; and *2*) these potential behavioral alterations could occur via dysregulations of the endocannabinoid system in selected brain regions regulating mood and cognition.

Diets were administered to healthy rats from pregnancy to adulthood and their consequences on measures of recognition and emotional memory, and depressive-, anxiety-, and psychotic-like behaviors were investigated in the adult male offspring. Subsequent biochemical analyses were carried out in the prefrontal cortex (PFC), hippocampus, and amygdala to check for possible alterations in: *1*) CB_1 receptor density and functionality; *2*) protein expression of endocannabinoid synthetic and degradative enzymes; and *3*) tissue levels of n-3- and n-6-derived endocannabinoids.

MATERIALS AND METHODS

Animals

Time-mated female Sprague-Dawley rats, starting from gestation day 1, were housed singularly in clear plastic cages on a 12 h light-dark cycle (lights on 8:00 AM) and in a temperature- (22 ± 2°C) and humidity-controlled environment (50 ±10%) with a plastic tube for environmental enrichment. All animals had free access to food and water. Newborn litters found up to 5:00 PM were considered to be born on that day [postnatal day (PND) 0]. On PND 1, all litters were reduced to a standard size of eight pups per litter (four males and four females). At birth, litter weights, the total number of pups, and the number of males and females were measured. On PND 21, pups were weaned. All experiments took place on the male adult offspring during the light phase and were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L. 2014/26) and the European Community directives regulating animal research (2010/63/EU). All efforts were made to minimize the number of animals used and their suffering.

Diet composition

Diets were provided by Dr. Piccioni Laboratories (Milan, Italy) and their composition was further assessed as follows.

Lipid extraction

Food pellets were separately mixed with a mixture of $CHCl_3$/methanol (2:1 v/v) at a ratio of 1:10 (w/v). After homogenization, samples were filtered and transferred to a new test tube. The extraction was done twice, and the two filtrates were combined together. A 0.9% NaCl solution was added to the recovered solution and centrifuged. The lower organic phase was removed, dried in vacuo, and stored at −80°C until analysis. Prior to analysis, dried lipid extracts were reconstituted in methanol.

GC-flame ionization detector analysis of fatty acid methyl esters

Lipids were derivatized to generate fatty acid methyl esters (FAMEs). The FAMEs were prepared as follows: 100 μl of extracted lipids were saponified with 1 ml of sodium methylate in methanol (0.5% w/v) at 100°C for 15 min. After cooling, 1 ml of boron trifluoride-methanol reagent was added and the solution was heated at 100°C for 15 min. The FAMEs were extracted by adding 1 ml of n-hexane, 4 ml of a saturated NaCl solution, and agitating manually for 2 min before a 5 min centrifugation (1,000 *g*). Finally, the n-hexane layer was transferred to a GC injector vial.

GC-flame ionization detector (FID) analyses were carried out on a GC2010 (Shimadzu, Milan, Italy) equipped with a split-splitless injector (260°C) and a FID. FAME separation was performed on a Supelcowax column (Sigma-Aldrich/Supelco, Bellefonte, PA; 30 m × 0.25 mm ID × 0.25 μm df). GC parameters were: initial pressure of carrier gas (helium) (constant linear velocity, 30 cm/s), 99.5 kPa; temperature program, 120–260°C at 3°C/min; injection volume, 1 μl; split ratio, 1:100. FID parameters were: temperature, 265°C; H_2 flow rate, 40 ml/min; air flow rate, 400 ml/min; make-up gas (He) flow rate, 50 ml/min. Data acquisition was performed using the GCsolution software.

An aliquot of the PFC and hippocampus lipid extract for each sample was mildly saponified and fatty acids were analyzed by HPLC (Agilent 1100 HPLC system with diode array detector; Palo Alto, CA) as previously described (19). Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. These spectra were acquired to confirm identification of the HPLC peaks (20).

Because saturated fatty acids are transparent to UV, after derivatization, they were measured as FAMEs by a gas chromatograph (Agilent Model 6890) as described (21).

Diet administration

Diets were administered according to the timeline reported in **Fig. 1A**. Time-mated Sprague-Dawley rats were fed a diet containing 6% fat in the form of peanut oil rich in LA (n-3-deficient diet), a diet containing 6% fat in the form of rapeseed oil rich in ALA (n-3-enriched diet), or a standard diet composed of 3% peanut oil and 3% rapeseed oil throughout gestation and lactation. Diets were isocaloric and did not differ with respect to the n-6 content. After weaning, the offspring received the same diet throughout the rest of their life to model a lifetime of n-3 deficiency or supplementation.

Behavioral tests

Classic and spatial versions of the novel object recognition test. The experimental apparatus used for the object recognition test was an open-field box (43 × 43 × 32 cm) made of Plexiglas placed in a dimly illuminated room. The experiment was performed and analyzed as previously described (22). Animals performed each test individually. Briefly, each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 min (familiarization phase). After an inter-trial interval of 3 min, one of the two familiar objects was replaced by a novel previously unseen object and rats were returned to the arena for the 5 min test phase. In the spatial variant of the test, the familiar object was placed in a different position compared with the familiarization phase; that is, a spatial cue was added in the test. During the test phase the time spent exploring the familiar object (Ef) and the time spent exploring the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows: $[(En - Ef)/(En + Ef)] \times 100$.

Social interaction test. The test was carried out as previously reported (22). During the test, each animal was allowed to freely explore an unfamiliar congener in an open-field box made of Plexiglas (60 × 60 × 60 cm) for 10 min and the total time spent in active social behaviors was recorded (as the sum of time spent in sniffing, following, grooming, mounting, and nosing during the test session) and the total number of aggressive episodes (attacking, biting, tail rattling, and aggressive grooming).

Forced swim test. Animals were tested in a modified version of the forced swim test (FST) with only the first session of swimming as previously reported (22, 23) to measure a preexisting behavioral deficit induced by diet administration.

Rats were forced to swim for 15 min inside a clear 50 cm tall, 20 cm diameter glass cylinder filled to 30 cm with 25°C water. During the test, the following parameters were monitored: immobility (time spent by the animal floating in the water making only those movements necessary to keep its head above the water), swimming (active swimming movements to the center of the cylinder), and climbing (forceful thrashing movements with forelimbs against the walls of the cylinder).

Elevated plus maze test. The test was performed as previously described (24). The elevated plus maze apparatus comprised two open arms (50 × 10 × 0 cm) and two closed arms (50 × 10 × 40 cm) that extended from a common central platform (10 × 10 cm). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. A video camera above the maze was connected to a television monitor connected to a videorecorder. Briefly, rats were individually placed on the central platform facing a closed arm for 5 min. The following parameters were analyzed: percent of time spent on the open arms, number of open arm entries, and total arm entries.

Inhibitory avoidance. The inhibitory avoidance apparatus consisted of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments separated by a sliding door. The starting compartment (31 cm long) was made of opaque white plastic and illuminated by a lamp; the shock compartment (60 cm long) was made of two dark electrifiable metal plates and was not illuminated. The procedure was carried out as previously described by Morena et al. (25). For training, rats were placed into the starting compartment facing away from the door and were allowed to explore the apparatus. After the rats stepped completely into the dark compartment, the sliding door was closed, and a single foot shock (0.4 mA) was delivered for 1 s. Animals were removed from the shock compartment 15 s after termination of the foot shock. Retention was tested 24 h later. On the retention test trial, rats were placed into the starting compartment, and the latency to re-enter the shock compartment with all four paws (maximum latency of 600 s) was recorded. Longer latencies were interpreted as indicating better retention.

Prepulse inhibition of startle reflex. The test was carried out as previously descripted (26). Briefly, each rat was placed in a prepulse inhibition (PPI) apparatus (Med Associates, St. Albans, VT) for a 5 min acclimatization period with a 70 dB background noise, which

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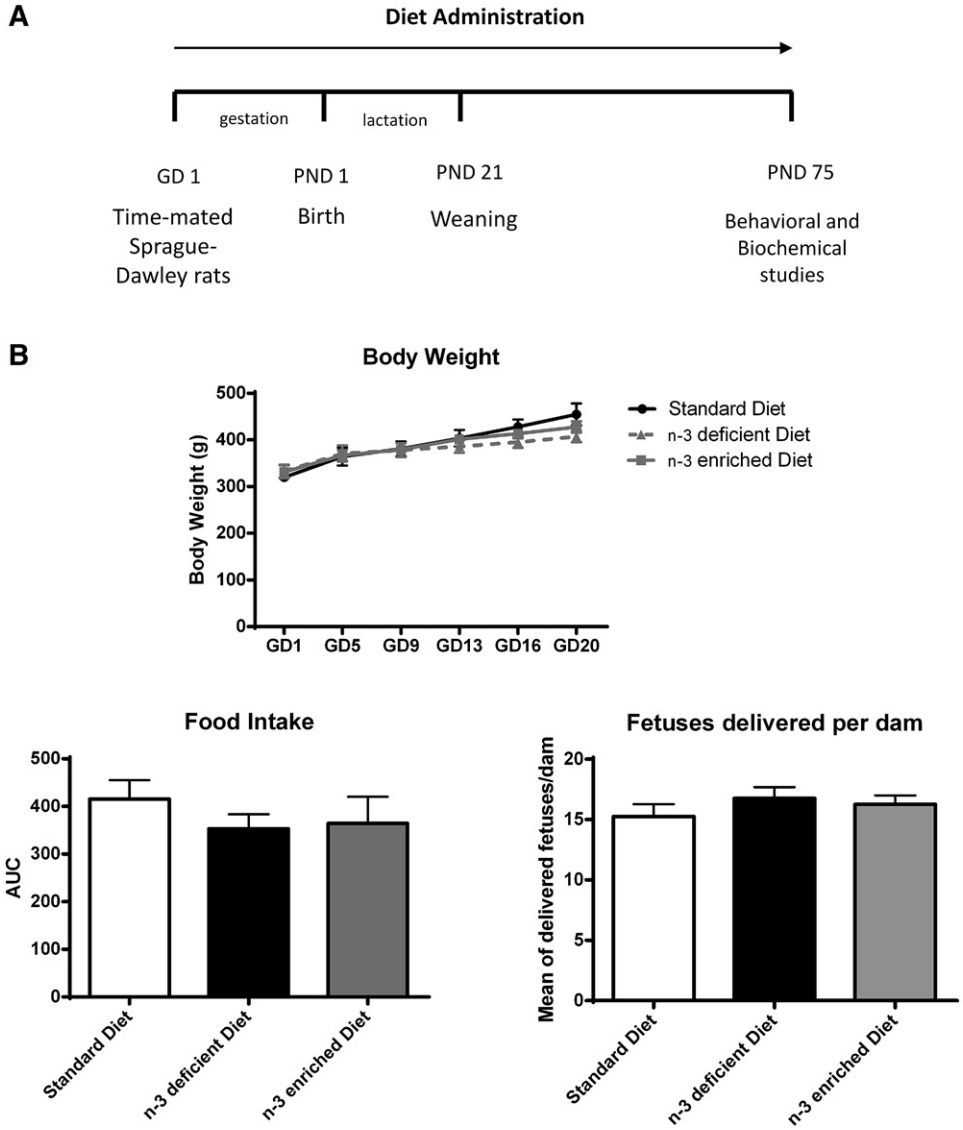

Fig. 1. A: Scheme of diet administration. Diets were administered to time-mated Sprague-Dawley rats throughout gestation and lactation. After weaning, the offspring received the same diet throughout the rest of their life to model a lifetime of n-3 deficiency or supplementation. B: Body weight gain, food intake in dams during gestation, and the number of fetuses delivered per dam. Data represent mean ± SEM of eight animals per group.

continued for the remainder of the session. Each session consisted of three consecutive sequences of trials. During the first and the third sequence, the rats were presented with five pulse-alone trials of 115 dB. The second sequence consisted of 50 trials in pseudo-random order, including 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76, or 82 dB prepulses (10 for each level of prepulse loudness), and eight no stimulus trials, where only the background noise was delivered. Intertrial intervals were selected randomly between 10 and 15 s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software. Percent PPI was calculated with the following formula: 100 − [(mean startle amplitude for prepulse − pulse trials/mean startle amplitude for pulse − alone trials) × 100].

Biochemical studies

The long-term effects of diet administration on components of the endocannabinoid system were evaluated in the adult male offspring (PND 75). Biochemical analyses were performed on animals that underwent novel object recognition (NOR), social interaction, and FSTs 24 h after the last test.

Autoradiographic-binding assays

Rats were euthanized and brains were rapidly removed, frozen in liquid nitrogen, and stored at −80°C until processing. Coronal sections (20 μm thick) were cut on a cryostat and mounted on gelatin-coated slides. The sections were stored at −80°C until processing.

[^{3}H]CP-55,940 receptor autoradiographic binding

The [^{3}H]CP-55,940 receptor autoradiographic binding was performed as previously described (27, 28).

CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography

This was determined as previously described (27, 28).

Image analysis

The intensity of the autoradiographic films was assessed by measuring the gray levels with an image analysis system consisting of a scanner connected to a computer running Microsoft Windows.

The images were analyzed using Image-Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD) as previously described (29).

Western blot analyses

For Western blot analyses, rats were euthanized and brains quickly removed. The cerebral areas of interest (PFC, hippocampus, and amygdala) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen, and stored at −80°C until processing.

The experiments were carried out as previously reported (30). Briefly, equal amounts of protein lysates (30 μg) were run on a 10% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membranes, blocked for 2 h at room temperature in 5% dry skimmed milk in TBS1x 0.1% Tween-20 before incubation overnight at 4°C with the primary antibody. The following primary antibodies were used: rabbit polyclonal anti-CB_1 (1:1,000; Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-NAPE-PLD (1:3,000; Cayman Chemical), rabbit polyclonal anti-FAAH (1:2,000; Cayman Chemical), goat polyclonal anti-DAGLα (1:1,000; Abcam, Cambridge, UK), rabbit polyclonal anti-MAGL (1:1,000; Cayman Chemical). Bound antibodies were detected with HRP-conjugated secondary anti-rabbit or anti-goat antibody (1:2,000 to 1:5,000; Chemicon International, Temecula, CA). For normalization, the blots were stripped with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) and reblotted with mouse anti-β-actin monoclonal antibody (1:10,000; Sigma-Aldrich, Italy) overnight at 4°C. Bound antibodies were visualized using Clarity Western ECL substrate (Bio-Rad Laboratories, Hercules, CA) and bands were detected with a GBOX XT camera (Syngene, Cambridge, UK). Optical density of the bands was quantified using Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD). The density of the bands was normalized to β-actin and expressed as arbitrary units.

Lipid extraction and endocannabinoid measurement

PFC, hippocampus, and amygdala were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl (50 mM, pH 7.4) (2:1:1, v/v) containing internal deuterated standards for AEA, PEA, OEA, docosahexaenoyl ethanolamide (DHA-EA), and 2-AG, quantification by isotope dilution [8-AEA, d4-PEA, d4-OEA, d4-DHA-EA, and d5-2-AG, (Cayman Chemical)], as well as 17:0-AE (Cayman Chemical) and 2-17:0-G (Larodan AB, Malmo, Sweden) for 18:2-EA, eicosapentaenoyl ethanolamide (EPA-EA), and 2-22:6-G measurement, respectively.

The lipid-containing organic phases were then purified by open bed chromatography on silica and fractions were obtained by eluting the column with 99:1, 90:10, and 50:50 (v/v) chloroform/methanol. Fractions eluted with chloroform/methanol 90:10 were collected, the excess solvent evaporated with a rotating evaporator, and aliquots analyzed by isotope dilution-LC/atmospheric pressure chemical ionization/MS carried out in the selected ion monitoring mode by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole mass spectrometer via a Shimadzu atmospheric pressure chemical ionization interface.

MS detection was performed by using values of *m/z* 356 and 348 (molecular ions + 1 for d8-AEA and AEA), *m/z* 304 and 300 (molecular ions + 1 for d4-PEA and PEA), *m/z* 330 and 326 (molecular ions + 1 for d4-OEA and OEA), *m/z* 376 and 372 (molecular ions + 1 for d4-DHA-EA and DHA-EA), *m/z* 384 and 379 (molecular ions +1 for d5-2-AG and 2-AG), *m/z* 346 (molecular ions + 1 for EPA-EA), *m/z* 324 (molecular ions + 1 for 18:2-EA), *m/z* 314 (molecular ions + 1 for 17:0-EA), *m/z* 403 (molecular ions + 1 for 2-22:6-G), and *m/z* 345 (molecular ions + 1 for 2-17:0-G).

AEA, PEA, OEA, DHA-EA, and 2-AG levels were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. EPA-EA, 18:2-EA, and 2-22:6-G were quantified against 17:0-EA and 2-22:6-G, respectively, the internal standards for which calibration curves had been constructed in pilot experiments. Lipid amounts expressed as picomoles were then normalized per gram or milligram of wet tissue.

Statistical analysis

Results are expressed as mean ± SEM and were analyzed by two- or one-way ANOVA, followed up by Bonferroni's post hoc test. The level of statistical significance was set at $P < 0.05$.

RESULTS

Diet composition

Table 1 reports the fatty acid composition of the diets used in this study. The n-3-deficient diet contained 6% fat in the form of peanut oil rich in LA (n-3:n-6 ratio = 1:23,9), the n-3-enriched diet contained 6% fat in the form of rapeseed oil rich in ALA (n-3:n-6 ratio = 1:4,5), and the standard diet was composed of 3% peanut oil and 3% rapeseed oil (n-3:n-6 ratio = 1:8,6).

Effect of lifelong dietary n-3 enrichment or deficiency on fatty acid contents in the PFC and hippocampus

To assess whether altered dietary ALA/LA ratio could actually affect brain fatty acid profile in the offspring, we quantified the fatty acid contents in the PFC and hippocampus of adult male rats after lifelong diet administration. The results are shown in **Tables 2, 3**. In the PFC (Table 2), consumption of n-3-enriched diets significantly decreased 18:2 n-6 and 20:3 n-6 contents by about 20% and 21% with respect to animals fed a standard diet. Administration of n-3-deficient diets significantly reduced 22:5 n-3 and 18:2 n-6 levels by about 32% and 35% compared with rats fed a standard diet. Lifelong dietary n-3 deficiency also significantly increased 20:3 n-6, 22:4 n-6, and 22:5 n-6 contents by about 60%, 10%, and 142%, respectively. Both dietary regimens did not affect DHA 22:6 n-3 and ARA 20:4 n-6 levels in this brain region. In the hippocampus (Table 3), n-3-enriched diets significantly increased 22:5 n-3 and 22:4 n-6 levels by about 29% and 15% compared with the standard diet. Significant decreases in DHA 22:6 n-3 (8%) and 22:5 n-6 (33%) levels were also present. Administration of n-3-deficient diets resulted in significant reductions by about 27% and 12% of 22:5 n-3 and DHA 22:6 n-3 contents, and in significant increases by about 9% and 108% of 22:4 n-6 and 22:5 n-6 levels. Neither enriched nor deficient diets altered hippocampal 18:2 n-6, 20:3 n-6, and ARA 20:4 n-6 levels.

TABLE 1. Fatty acid composition of the dietary lipids (relative percent of total fatty acids)

	Standard Diet	n-3-Enriched Diet	n-3-Deficient Diet
LA	32.0	27.5	33.3
ALA	3.7	6.0	1.4
Monounsaturated fats	35.7	48.3	38.7
Polyunsaturated fats	35.7	33.6	34.7
n-3:n-6 ratio	1:8.6	1:4.5	1:23.9
Energy content (kcal/g)	4.09	4.05	4.12

All diets were isocaloric, had the same amount of n-6, and differ only for n-3 content.

TABLE 2. Effect of lifelong administration of n-3-enriched or -deficient diets on the fatty acid profile in the PFC of adult rats

	Standard Diet	n-3-Enriched Diet	n-3-Deficient Diet
22:5 n-3	0.752 ± 0.024	0.508 ± 0.040[b]	0.883 ± 0.053
DHA 22:6 n-3	12.745 ± 0.167	11.595 ± 0.480	13.571 ± 0.288
18:2 n-6	2.319 ± 0.136	1.496 ± 0.039[a]	1.845 ± 0.042[c]
20:3 n-6	0.418 ± 0.017	0.667 ± 0.008[a]	0.328 ± 0.016[b]
ARA 20:4 n-6	12.041 ± 0.259	12.080 ± 0.344	12.343 ± 0.081
22:4 n-6	2.197 ± 0.048	2.427 ± 0.068[b]	2.303 ± 0.022
22:5 n-6	0.617 ± 0.016	1.492 ± 0.141[a]	0.422 ± 0.010

Data represent mean ± SEM for four rats per group and are expressed as nanomoles per milligram of lipid.
[a] $P < 0.001$ versus standard diet.
[b] $P < 0.01$ versus standard diet.
[c] $P < 0.05$ versus standard diet.

Effect of diet administration on body weight, food intake, and pregnancy outcome in time-mated Sprague-Dawley rats

As shown in Fig. 1B, administration of n-3-deficient and -enriched diets during gestation did not affect body weight gain and food intake in time-mated rats when compared with controls fed a standard chow diet. Furthermore, the number of fetuses delivered per dam was similar for all the diets considered and no changes in body weight gain were observed in the offspring (data not shown).

Behavioral consequences of lifelong dietary n-3 imbalances in the male offspring

Male adult (PND 75) offspring were submitted to a series of behavioral tests to evaluate the long-term consequences of dietary n-3 imbalances from pregnancy on cognitive performance, emotional reactivity, and psychotic-like symptoms.

Figure 2 shows the consequences of diet administration on recognition and emotional memory, as measured through the NOR test (Fig. 2A) and the inhibitory avoidance task (Fig. 2B), respectively.

One-way ANOVA showed significant effects of diet administration both in the classic ($F_{2,17} = 9.392$; $P = 0.0035$) and in the spatial ($F_{2,17} = 5.892$; $P = 0.0121$) versions of the NOR test. Lifelong administration of both n-3-deficient and -enriched diets resulted in impaired cognitive performance in the classic version of the NOR test in the adult male offspring, as stated by the significant reductions of the discrimination index by about 76% and 60%, respectively, compared with controls fed a standard diet. A similar effect was also observed in the spatial version of the test, the discrimination index being reduced by about 64% and 50% in both experimental groups.

In contrast, diet administration did not significantly affect emotional memory in the inhibitory avoidance task, as demonstrated by the presence of similar retention performances in all the experimental groups.

Figure 3 reports the effects of diet administration on measures of depressive- and anxiety-like behaviors, such as the social interaction test (Fig. 3A), the FST (Fig. 3B), and the elevated plus maze (Fig. 3C), as well as on psychotic-like symptoms, through the PPI test (Fig. 3D).

Statistical analysis revealed a significant effect of diets in the social interaction test ($F_{2,17} = 6.628$; $P = 0.0062$) and FST (immobility: $F_{2,17} = 4.468$, $P = 0.0257$; climbing: $F_{2,17} = 7.512$, $P = 0.0039$). Administration of n-3-enriched diets induced a significant reduction in the time spent in active social behaviors in the 10 min test session by about 52% when compared with controls. Similarly, in the FST, rats fed n-3-enriched diet displayed a significant increase by about 60% in the time spent in immobility during the 15 min test session, paralleled by a significant 36% reduction of the time spent in climbing activity. In contrast, n-3-deficient diet administration did not alter the animals' behavior in the social interaction test and FST.

Conversely, no significant effects were instead observed in the elevated plus maze test, because administration of n-3-deficient and -enriched diets did not change the time spent in the open arms and the percentage of open arm entries with respect to controls. This effect did not appear to be due to motoric effects, as there was no change in total arm entries (data not shown).

Finally, dietary fatty acid composition had no effect on PPI responses in the adult offspring.

Behavioral consequences of n-3 enrichment or deficiency restricted to gestation and lactation

In order to dissect between the behavioral effect of lifelong or perinatal administration of n-3-deficient or -enriched diets, we performed a separate experiment in which diets were given either lifelong or during gestation and lactation only. The offspring belonging to all experimental groups were then tested for behavior at adulthood, i.e., PND 75.

Figure 4 represents the effects of lifelong or perinatal diet administration on recognition memory (Fig. 4B) and depressive-like behaviors, as measured through the social interaction test (Fig. 4C) and FST (Fig. 4D).

Statistical analysis revealed the main effects of diet, time, and diet × time interaction in the NOR test (diet: $F_{2,30} = 48.61$, $P < 0.0001$; time: $F_{1,30} = 12.92$, $P = 0.0011$; diet × time interaction: $F_{2,30} = 11.54$, $P = 0.0002$). As expected, lifelong administration of both n-3-deficient or -enriched diets significantly reduced the discrimination index in the adult offspring by about 70% with respect to animals fed a standard diet. Interestingly, n-3 deprivation during gestation and lactation was sufficient to induce a significant impairment of recognition memory in adult animals. In contrast, administration of n-3-enriched diet restricted to gestation and lactation did not alter recognition memory at adulthood.

TABLE 3. Effect of lifelong administration of n-3-enriched or -deficient diets on the fatty acid profile in the hippocampus of adult rats

	Standard Diet	n-3-Enriched Diet	n-3-Deficient Diet
22:5 n-3	0.248 ± 0.014	0.180 ± 0.009[c]	0.319 ± 0.044[c]
DHA 22:6 n-3	11.394 ± 0.043	10.048 ± 0.199[a]	10.423 ± 0.164[b]
18:2 n-6	1.444 ± 0.136	1.302 ± 0.095	1.651 ± 0.082
20:3 n-6	0.522 ± 0.033	0.631 ± 0.031	0.442 ± 0.019
ARA 20:4 n-6	12.665 ± 0.232	12.733 ± 0.316	12.218 ± 0.353
22:4 n-6	2.284 ± 0.045	2.491 ± 0.044[c]	2.641 ± 0.089[b]
22:5 n-6	0.579 ± 0.028	1.202 ± 0.041[a]	0.385 ± 0.023[b]

Data represent mean ± SEM for four rats per group and are expressed as nanomoles per milligram of lipid.
[a] $P < 0.001$ versus standard diet.
[b] $P < 0.01$ versus standard diet.
[c] $P < 0.05$ versus standard diet.

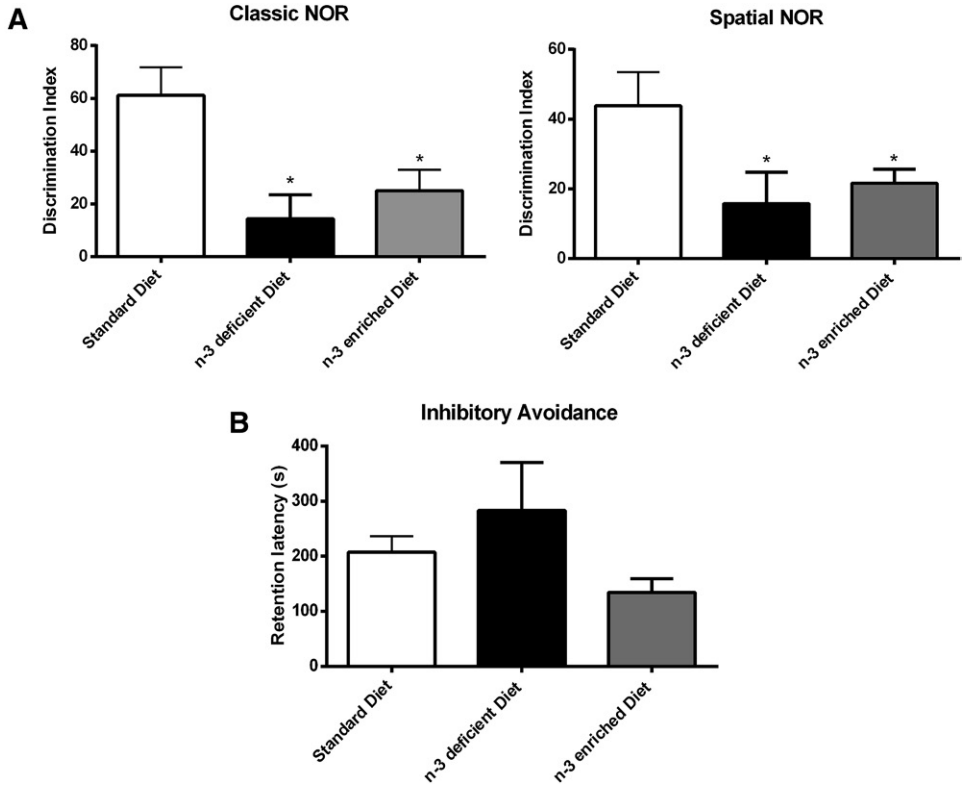

Fig. 2. Effect of diet administration on nonemotional and emotional memories in the adult male offspring. A: Cognitive performance in the NOR test in the classic (left panel) and in the spatial (right panel) versions of the task. The discrimination index was calculated as follows: [(En − Ef)/(En + Ef)] × 100. B: Inhibitory avoidance retention latencies in seconds. Rats were given foot shock in compartment A and 48 h later they were tested for retention latencies in the same apparatus. Data represent mean ± SEM of eight animals per group for the NOR test and mean ± SEM of 10 to 11 animals per group for the inhibitory avoidance task. *$P < 0.05$ versus standard diet (Bonferroni's post hoc test).

In the social interaction test, two-way ANOVA showed significant main effects of diet, time, and diet × time interaction (diet: $F_{2,30} = 3.738$, $P = 0.0355$; time: $F_{1,30} = 12.01$, $P = 0.0016$; diet × time interaction: $F_{2,30} = 20.96$, $P < 0.0001$). Lifelong administration of n-3-enriched diets induced a significant reduction in the time spent in active social behaviors by about 50% when compared with controls; whereas, lifelong administration of n-3-deficient diet did not alter sociability in the long-term. Interestingly, n-3-enriched diets given during gestation and lactation only did not alter social behaviors in the adult offspring. A similar effect was also observed in the FST. Indeed, statistical analysis revealed the main effects of diet, time, and diet × time interaction in the time spent in immobility (diet: $F_{2,30} = 8.016$, $P = 0.0016$; time: $F_{1,30} = 11.46$, $P = 0.0020$; diet × time interaction: $F_{2,30} = 17.54$, $P < 0.0001$) and climbing activities (diet: $F_{2,30} = 11.33$, $P = 0.0002$; time: $F_{1,30} = 9.502$, $P = 0.0044$; diet × time interaction: $F_{2,30} = 12.73$, $P < 0.0001$). Lifelong administration of n-3-enriched diets led to a significant increase, by about 50%, in the time spent in immobility, paralleled by a significant 35% reduction of the time spent in climbing activity, in the adult offspring. In contrast, no alterations were observed in adult animals that were fed n-3-enriched diets only during perinatal life. Finally, neither lifelong nor perinatal administration of n-3-deficient diets altered the animals' behavior in the FSTs.

Diet-induced changes in the endocannabinoid system in the PFC, hippocampus, and amygdala of adult male offspring

Biochemical analyses were carried out in the brains of the adult offspring in order to determine whether lifelong dietary n-3 deficiency or supplementation could impact the endocannabinoid system in the long-term. Specifically, CB_1 receptor density and functionality and protein expression of endocannabinoid synthetic and degradative enzymes (NAPE-PLD, DAGLα, FAAH, and MAGL), as well as the levels of n-3- and n-6-derived endocannabinoids, were monitored in the PFC, hippocampus, and amygdala, brain regions closely involved in the modulation of cognition and emotionality.

Figure 5 reports the effect of diet administration on the endocannabinoid system in the PFC of adult male rats.

The composition of dietary fatty acids did not alter CB_1 receptor expression and G protein coupling within this brain area (Fig. 5A). In contrast, statistical analysis revealed that both n-3 deficiency and supplementation significantly affected the expression of the degradative enzymes for endocannabinoids, FAAH ($F_{2,14} = 7.450$; $P = 0.0241$) and MAGL ($F_{2,14} = 11.10$; $P = 0.0037$). The n-3 supplementation significantly increased the expression of both FAAH and MAGL by about 50% and 180%, respectively, when compared with control rats. Similarly, administration of an n-3-deficient

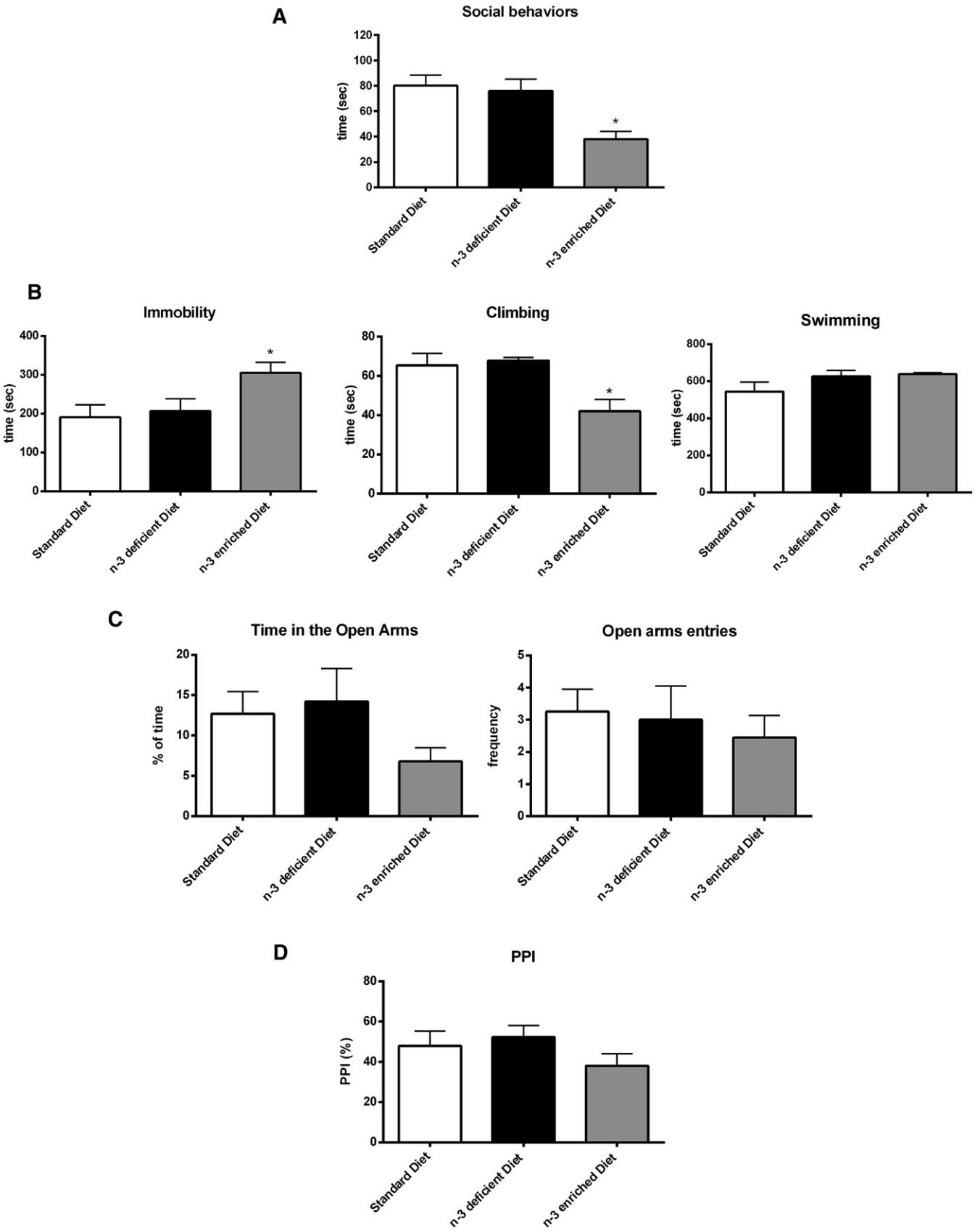

Fig. 3. Effect of diet administration on depressive- and anxiety-like behaviors and psychotic-like signs in the adult male offspring. A: Active social behaviors in the social interaction test. B: Time spent in immobility, climbing, and swimming in the FST. C: Time spent in the open arms and open arm entries in the elevated plus maze test. D: PPI response. Data represent mean ± SEM of eight animals per group for the social interaction and FSTs and mean ± SEM of 10 to 11 animals per group for the elevated plus maze and PPI tasks. $*P < 0.05$ versus standard diet (Bonferroni's post hoc test).

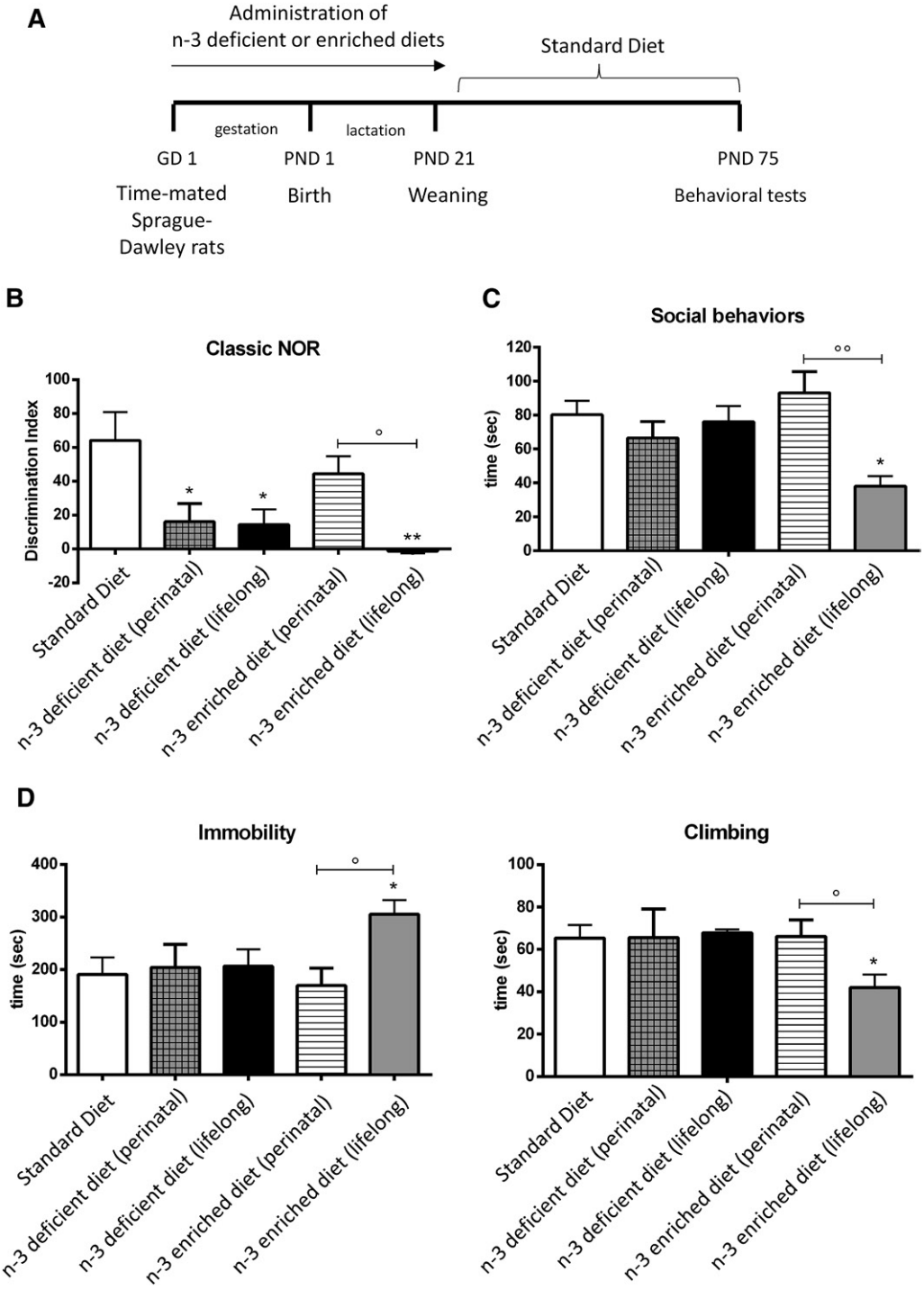

Fig. 4. Long-term consequences of lifelong or perinatal administration of n-3-deficient or -enriched diets. Experimental procedure (A), recognition memory (B), and depressive-like behaviors (C and D) as measured through the social interaction test and forced swim test. Data represent mean ± SEM of six animals per group. $^{**}P < 0.01$; $^{*}P < 0.05$ versus standard diet; $^{\circ\circ}P < 0.01$, $^{\circ}P < 0.05$ versus lifelong administration of n-3-enriched diet (Bonferroni's post hoc test).

diet resulted in a significant 200% increase in MAGL expression without altering FAAH protein levels. No alterations on NAPE-PLD and DAGLα expression were observed in any of the experimental groups considered (Fig. 5B). Changes in degradative enzymes were paralleled by significant alterations of endocannabinoid levels within the PFC (**Fig. 6**). One-way ANOVA showed that modifications of dietary n-3 intake triggered significant changes in the levels of AEA ($F_{2,9} = 5.824$; $P = 0.0238$) and 2-AG ($F_{2,9} = 5.377$; $P = 0.0291$), as well as the n-3 derivative, MAG 2-22:6 ($F_{2,9} = 8.389$; $P = 0.0151$). Levels of AEA and 2-AG significantly decreased by about 70% and 40% in the PFC after administration of n-3-enriched diets. A significant 50% reduction of MAG 2-22:6 was also observed in this experimental group.

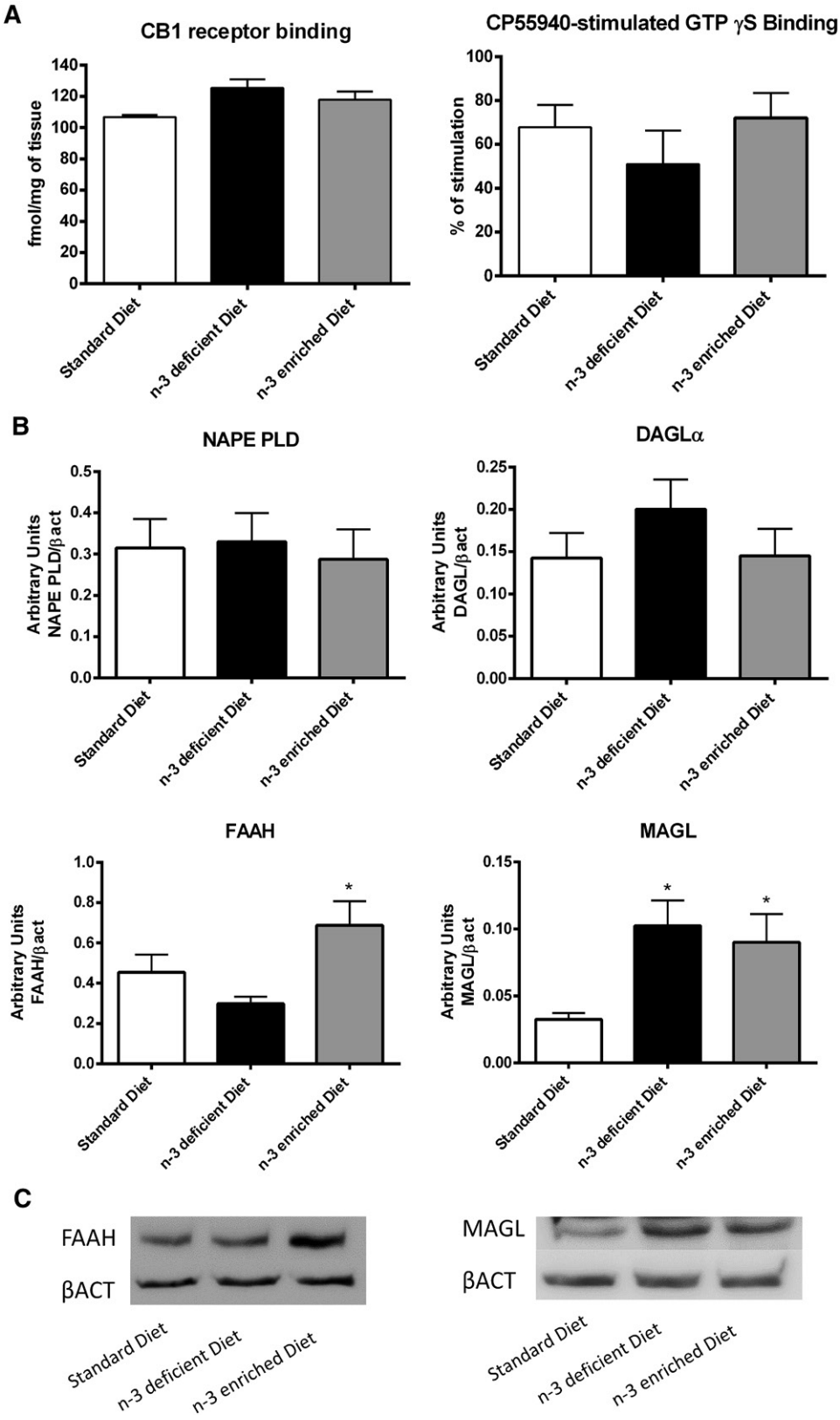

Fig. 5. Effect of dietary n-3 intake on the endocannabinoid system within the PFC of adult male offspring. A: [^{3}H]CP-55,940 receptor binding in autoradiography (left panel) and CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography (right panel). Results are expressed as mean ± SEM of four animals per group. B: Protein levels of synthetic and degradative enzymes for endocannabinoids. Data are expressed as arbitrary units and represent mean ± SEM of five animals per group. *$P < 0.05$ versus standard diet (Bonferroni's post hoc test). C: Representative Western blot images of FAAH and MAGL. β-Actin was probed as loading control.

In contrast, administration of n-3-deficient diets led to reductions of 2-AG (−30%) and MAG 2-22:6 (−40%), without affecting AEA levels. PEA, OEA, DHA-EA, EPA-EA, and 18:2-EA levels were not altered by the different dietary conditions.

Alterations of the endocannabinoid system were also present in the hippocampus of adult male rats after administration of both n-3-deficient and -enriched diets (**Figs. 7, 8**). Administration of both n-3-deficient and -enriched diets significantly reduced CB_1 receptor functionality by about 30% within the hippocampus of the adult male offspring ($F_{2,9} = 4.452$; $P = 0.0194$), whereas no effect was observed on CB_1 receptor density (Fig. 7A). Diet administration also affected the expression of the enzymes responsible for endocannabinoid synthesis and degradation (Fig. 7B). Specifically, statistical analysis showed an effect of dietary n-3 intake on NAPE-PLD ($F_{2,14} = 5.746$; $P = 0.0247$) and MAGL ($F_{2,14} = 7.911$; $P = 0.0127$) expression. Lifelong administration of n-3-enriched diets significantly reduced NAPE-PLD protein levels by about 40% and increased MAGL expression by about 60% when compared with controls fed a standard diet. No alterations were observed after administration of n-3 deficient diets.

As shown in Fig. 8, diet administration also affected endocannabinoid levels within the hippocampus (AEA: $F_{2,9} = 5.245$, $P = 0.0277$; 2-AG: $F_{2,9} = 4.467$, $P = 0.0449$; MAG 2-22:6: $F_{2,9} = 8.114$, $P = 0.0097$; DHA-EA: $F_{2,9} = 11.55$, $P = 0.0033$). Levels of AEA and 2-AG decreased in this brain region after administration of n-3-enriched diet. A similar decrease was also observed for MAG 2-22:6, its levels being reduced by about 60% after administration of the enriched diet. In contrast, n-3 enrichment resulted in a significant increase, by about 60%, of the levels of the n-3 derivative, DHA-EA. No significant changes were observed in animals that underwent administration of n-3-deficient diets and dietary n-3 content did not affect PEA, OEA, EPA-EA, and 18:2-EA levels.

Finally, in the amygdala, dietary fatty acid composition did not affect CB_1 receptor density and functionality or AEA, 2-AG, PEA, OEA levels within this brain region (data not shown).

DISCUSSION

The present results indicate that sustained imbalances in dietary ALA from pregnancy have long-term consequences on adult offspring behavior in terms of cognitive and emotional functions. Specifically, we found that lifelong administration of both ALA-deficient and -enriched diets

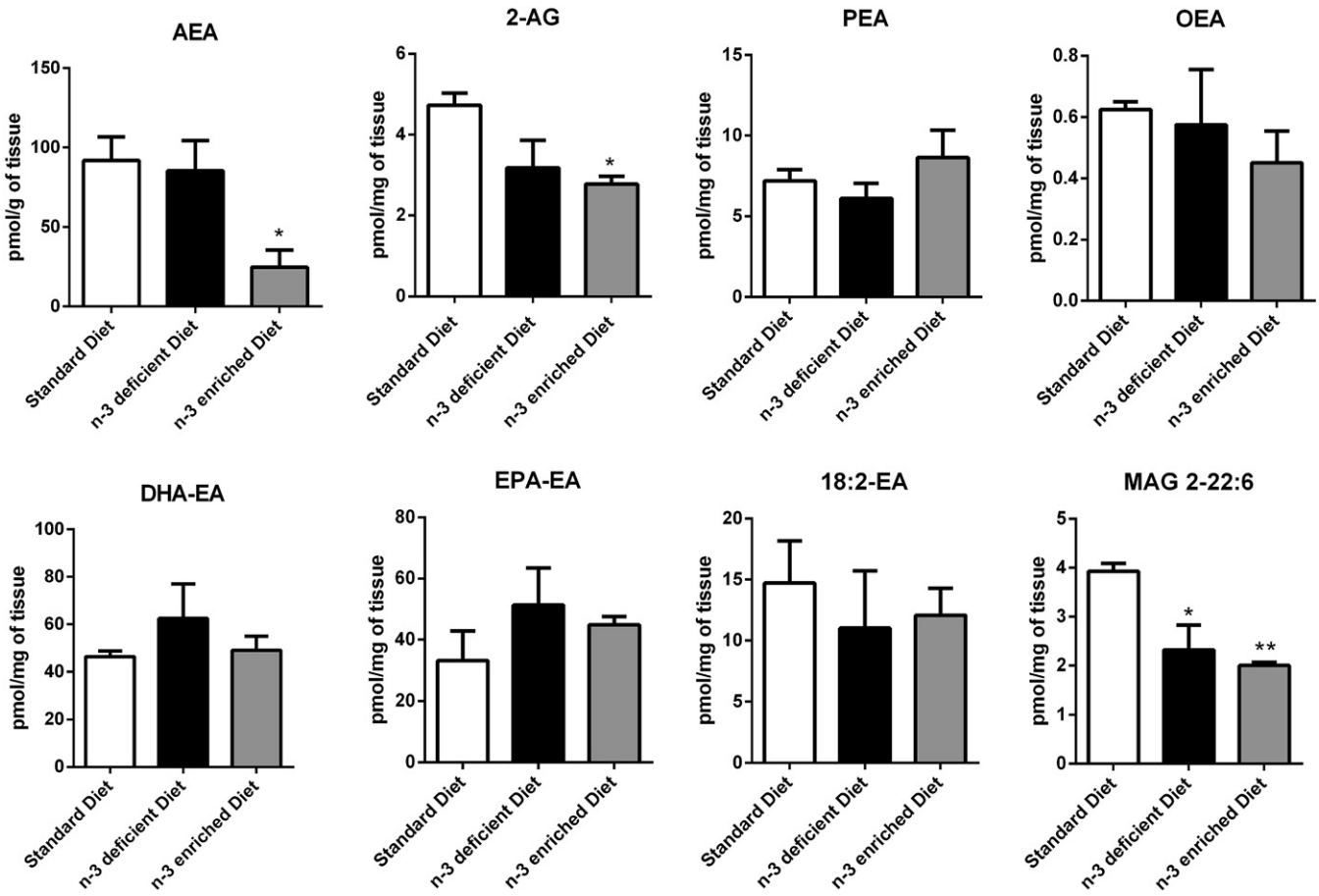

Fig. 6. Tissue concentrations of n-6-derived endocannabinoids (AEA, 2-AG, PEA, OEA) and n-3-derivatives (DHA-EA, EPA-EA, 18:2-EA, MAG 2-22:6) in the PFC of adult male rats after lifelong administration of deficient and enriched diets. Data are expressed as the mean ± SEM of four animals per group. *$P < 0.05$; **$P < 0.01$ versus standard diet (Bonferroni's post hoc test).

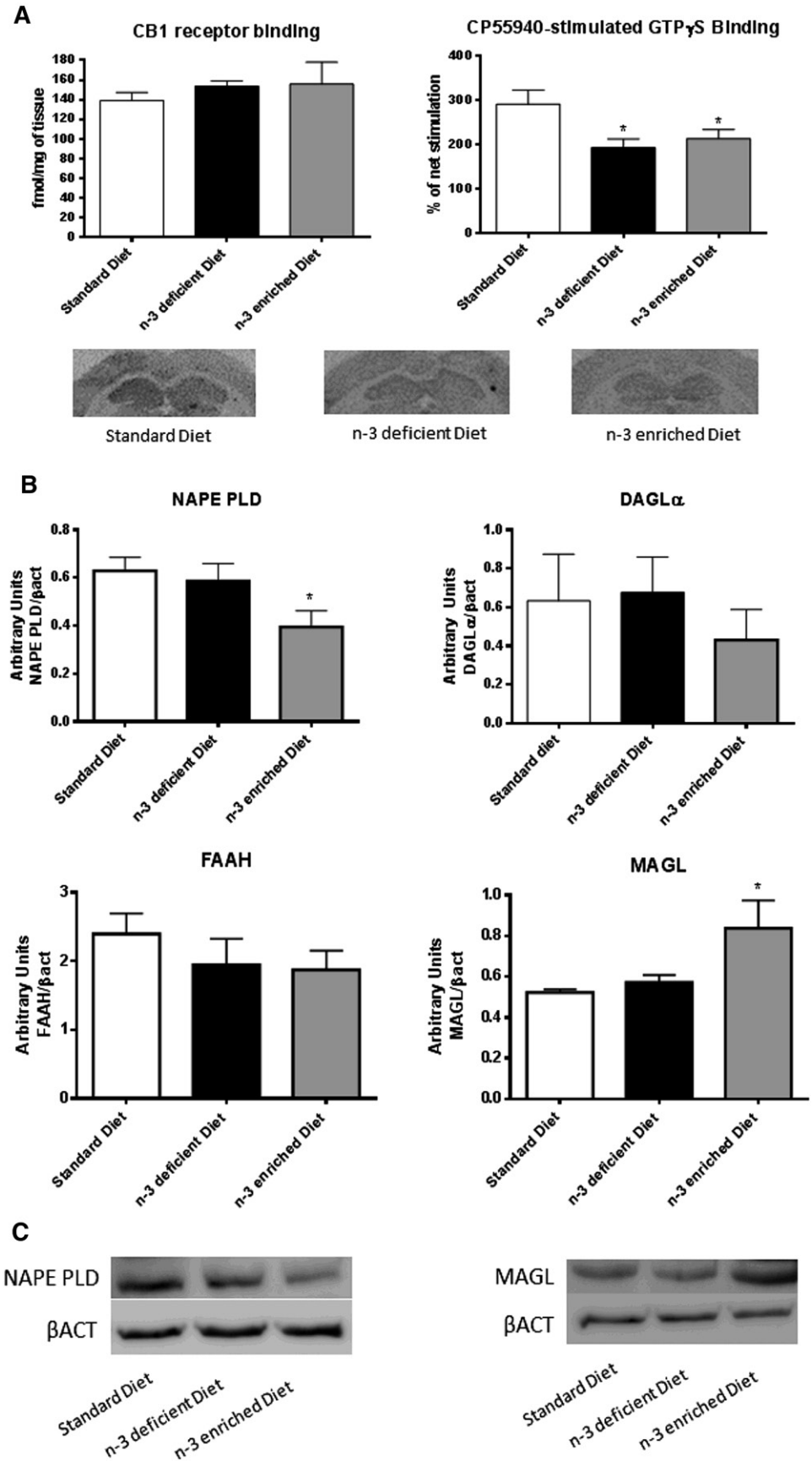

Fig. 7. Effect of dietary n-3 intake on the endocannabinoid system within the hippocampus of adult male offspring. A: [^{3}H]CP-55,940 receptor binding in autoradiography (left panel) and CP-55,940-stimulated [^{35}S] GTPγS binding in autoradiography (right panel). Results are expressed as mean ± SEM of four animals per

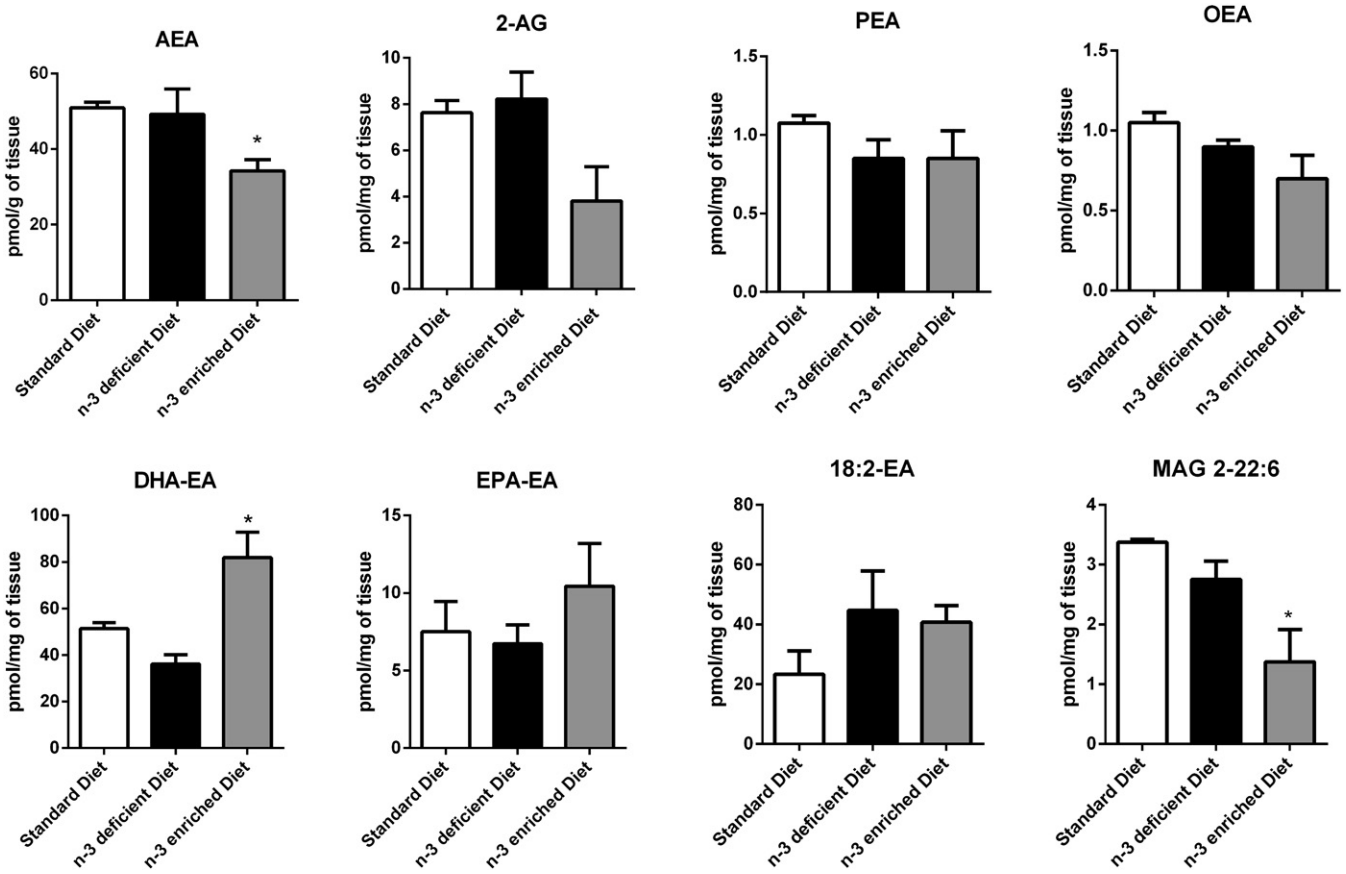

Fig. 8. Tissue concentrations of n-6-derived endocannabinoids (AEA, 2-AG, PEA, OEA) and n-3-derivatives (DHA-EA, EPA-EA, 18:2-EA, MAG 2-22:6) in the hippocampus of adult male rats after lifelong administration of deficient and enriched diets. Data are expressed as the mean ± SEM of four animals per group. $^*P < 0.05$ versus standard diet (Bonferroni's post hoc test).

induced lasting short-term memory deficits in the NOR test; whereas, dietary ALA enrichment also resulted in abnormal emotional reactivity in the social interaction test and FST in adult male rats with respect to animals fed a standard diet, balanced in n-3 content. Accordingly, we found a decrease of DHA in the PFC of rats fed both ALA-deficient and -enriched diets with respect to control diet. The decrease of DHA in the brains of rats fed an ALA-deficient diet has already been described in mice (17, 18). However, the decrease of DHA in the brains of rats fed the ALA-enriched diet is also not surprising, because it has been shown that high dietary ALA inhibits delta 6 desaturase, impairing the formation of the direct precursor of DHA, c24:6n-3 fatty acid (31). In fact, we found an increase of the other precursor, 22:5n-3. Interestingly, in our experimental conditions, we didn't detect any change in AA content in contrast with previous findings in mice (17, 18). Thus, we may suggest that long-term feeding of imbalanced n-3/n-6 ratio diets from the gestational period may result in a physiological response to maintain a steady AA concentration in the brain by modulating its metabolism, including endocannabinoid biosynthetic and degrading enzymes.

The n-3 and n-6 LCPUFAs are essential elements in the assembly, maturation, and physiological function of neuronal structures, and optimal neuronal development is highly dependent on the supply of LCPUFAs (2). Thus, dysregulation in brain n-3 LCPUFA content could adversely affect brain maturation and neuronal activity, and a diet balanced in n-6 and n-3 contents should be recommended to ensure normal brain functions. Accordingly, our data demonstrate that when the optimal n-6/n-3 ratio is lost, both following dietary ALA deficiency and enrichment, long-term behavioral abnormalities in cognition and emotionality arise.

However, literature data on the effects of dietary n-3 deficiency and supplementation are often conflicting. Impairment in several aspects of learning and memory has been reported as a consequence of pre- and postnatal dietary n-3 deficiency (32–35), while a transient or maternal n-3 PUFA-deficient diet has been reported to induce depressive- and anxiety-like symptoms, as well as abnormal social behavior in adult offspring (17, 18, 36–40). Notably, other studies found opposite or no effects of different n-3-deficient and -supplemented diets on behavior (41–46). Interestingly, the discriminant is DHA concentration.

group. B: Protein levels of synthetic and degradative enzymes for endocannabinoids. Data are expressed as arbitrary units and represent mean ± SEM of five animals per group. $^*P < 0.05$ versus standard diet (Bonferroni's post hoc test). C: Representative Western blot images of NAPE-PLD and MAGL. β-Actin was probed as loading control.

In fact, it is well-established that dietary ALA deficiency results in DHA brain deficiency; whereas, the most efficient strategy to increase DHA levels in brain is its dietary intake, more efficiently in the phospholipid form, as in Krill oil (21). In our hands, lifelong administration of n-3-deficient diets impaired short-term memory performance in adult offspring when compared with controls fed a standard diet without inducing any depressive- or anxiety-like symptom.

Discrepancies in the literature data could be attributed to differences in diet compositions, such as the type of n-3 fatty acid and protocols of administration used. Indeed, to reach an imbalanced n-6/n-3 ratio in the n-3-deficient diets, in some studies overall n-6 content was markedly increased (17, 18, 39, 45), while in others n-3 fatty acids were completed depleted (37, 40). Moreover, great variability in the time window and duration of administration was also present. Diets were administered either starting before mating (42, 46), during gestation and lactation (37, 43, 45), after weaning (38), from pregnancy throughout adulthood (17, 18, 44), or across consecutive generations (41). Our findings further highlight the importance of the time window of administration in determining the detrimental behavioral effects of dietary ALA enrichment or deficiency. In fact, when isocaloric diets enriched or deficient in ALA, but with similar overall LA, were administered from pregnancy throughout adulthood, both dietary regimens led to significant behavioral abnormalities in the adult male offspring. In contrast, when ALA enrichment or deficiency was restricted to gestation and lactation, a different picture was observed. In fact, while administration of n-3-deficient diets restricted to this period was sufficient to impair recognition memory in adult animals, no behavioral alterations in terms of either cognitive deficits or depressive-like behaviors were observed in the adult offspring after administration of n-3-enriched diets. Thus, it appears that the perinatal phases are the more sensitive period for the negative consequences of n-3 deficiency on cognition; whereas, the adverse effects of n-3 enrichment arise only when enriched diets are administered for a longer period (i.e., lifelong).

Among others, one of the added values of our study is that it also investigated the effects of lifelong ALA enrichment per se in comparison to a standard diet. This finding further underlines the importance of the right PUFA composition for proper brain development and behavior. Thus, clinical studies investigating the efficacy of n-3 PUFAs for the treatment of neurological conditions should take into account the initial n-3 LCPUFA status of the subjects in order to determine the optimal dose required to achieve any potential benefit. Indeed, n-3 LCPUFA supplementation could exert some benefits by restoring a balanced n-6/n-3 ratio in those subjects in which n-3 intake is deficient.

In the context of the possible molecular mechanisms involved in the observed behavioral alterations, the endocannabinoid system could represent an ideal candidate to mediate the effects of dietary fatty acids on brain and behavior (47).

Endocannabinoids include the fatty acid ethanolamides, AEA, OEA, and PEA, as well as the ester, 2-AG, biosynthesized from PUFAs of the n-6 family, and those biosynthesized from n-3 PUFAs, such as EPA-EA and DHA-EA (48–50). Endocannabinoids are important regulators of synaptic function (16). They suppress neurotransmitter release by acting as retrograde messengers at presynaptic CB_1 receptors. Retrograde endocannabinoid signaling mediates short-term forms of synaptic plasticity, as well as presynaptic forms of long-term depression at both excitatory and inhibitory synapses (51). Moreover, the endocannabinoid system has been shown to play a central role in the modulation of mood and cognition both at adulthood and during critical developmental stages (52), and it is engaged in food addiction (53).

In the present paper, we demonstrated that dietary ALA profoundly alters the brain endocannabinoid system, in terms of both endocannabinoid levels as well as their synthetic and degrading enzymes. Both ALA deficiency and enrichment increase MAGL levels in the PFC and hippocampus with respect to rats fed the standard diet and this is associated with reductions of 2-AG and MAG 2-22:6 levels. In the same brain regions, lifelong administration of ALA-enriched diets also reduces AEA levels, possibly via increased AEA degradation by FAAH in the PFC and reduced AEA synthesis by NAPE-PLD in the hippocampus. Intriguingly, ALA-deficient and -enriched diets alter AEA and 2-AG contents without affecting the concentrations of "entourage compounds", such as PEA and OEA. The observation that whenever MAGL expression is increased, reductions of both 2-AG and MAG 2-22:6 are found, strongly suggests that MAGL could also be responsible for the degradation of this n-3-derived endocannabinoid. As mentioned above, the modulation of biosynthetic and degrading enzymes of AEA and 2-AG, as well of MAG 2-22:6, might be envisaged as a physiological mechanism to maintain brain AA and DHA steady concentrations.

As recent evidence indicates that MAGL deletion and subsequent elevation of 2-AG levels enhance memory in the NOR test (54), it is tempting to speculate that the cognitive impairments induced by both ALA-deficient and -enriched diets, other than DHA decrease, could possibly be a consequence of deficient 2-AG signaling within the PFC and hippocampus. Conversely, reduced AEA levels, as well as FAAH dysfunction, have been linked to depression both in animals and humans (55–58), consistent with the phenotype observed in rats administered with ALA-enriched diets. Of course, these hypotheses are merely speculative at this time, and further studies are needed to clearly establish any specific correlation. Of note, the expression of endocannabinoid system components can be regulated by epigenetic mechanisms (59), and food intake has, indeed, been shown to modulate CB_1 expression via DNA methylation (47). Therefore, it is tempting to speculate that the modulation of endocannabinoid system protein expression by ALA shown here occurs through epigenetic mechanisms.

Finally, both diets did not alter the animals' performance in the inhibitory avoidance task and, accordingly, no changes in CB_1 receptor and endocannabinoid content

were observed in the amygdala, a key area involved in the modulation of this behavioral response.

Endocannabinoids, both n-6 and n-3 derived, can bind and activate CB_1 receptors, although with different affinity (60, 61). Both ALA-deficient and -enriched diets reduce CB_1 receptor/G protein coupling in the hippocampus, whereas no changes were present in the PFC and amygdala. Accordingly, it has recently been shown that lifelong administration of diets rich in ALA and LA reduced ERK1/2 phosphorylation in the hippocampus after treatment with WIN55212-2 (18), suggesting an impairment in the CB_1 signaling pathway after administration of both ALA-enriched and -deficient diets. In our hands, both ALA-enriched and -deficient diets resulted in CB_1 receptor desensitization in the presence of decreased or unchanged 2-AG and AEA contents in the hippocampus. Given that membrane lipid composition has been demonstrated to affect CB_1 cannabinoid receptor binding and signaling (62), it is possible that the effect of diets on CB_1 receptor functionality could be the consequence of alterations of brain fatty acid composition triggered by dietary PUFAs.

Taken together, these findings support the critical role of n-3 PUFAs in brain maturation and functioning by comparing the effects of diets enriched or deficient in ALA to those of a standard diet, thus highlighting the importance of a balanced diet that ensures adequate intake of key nutrients for mental health. More interestingly, our data support a link between dietary intake of ALA, brain endocannabinoid system, and behavior, indicating that dietary ALA is a sufficient condition for altering the endocannabinoid system in brain regions modulating mood and cognition. Dietary n-3 PUFAs act as homeostatic regulators of the endocannabinoid system and adequate levels of dietary n-3 fatty acids are required for proper endocannabinoid signaling. Overall, modulation of the endocannabinoid system by dietary n-3 PUFAs could represent an interesting approach for future clinical investigations. However, attention should be paid when prescribing n-3 PUFA supplementation without specifying the type of n-3 PUFA as adjuvant treatment for psychiatric conditions, as its effect in terms of the CB_1 receptor and/or endocannabinoids can be difficult to predict, especially after chronic intakes.

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